Freshwater Biology

Context dependency of infectious disease: the cyanobacterium Microcystis aeruginosa decreases White Bacterial Disease in Daphnia magna.

Journal:	*Freshwater Biology*
Manuscript ID:	FWB-P-Apr-13-0225.R2
Manuscript Type:	Standard Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Coopman, Marlies; KULeuven-kulak, Science and Technology, Aquatic Biology Muylaert, Koenraad; KULeuven-kulak, Science and Technology, Aquatic Biology Lange, Benjamin; KULeuven-kulak, Science and Technology, Aquatic Biology Reyserhove, Lien; KULeuven-kulak, Science and Technology, Aquatic Biology Decaestecker, Ellen; KULeuven-kulak, Science and Technology, Aquatic Biology
Keywords:	Zooplankton < Taxonomic Group / Assemblage, Parasites / pathogens < Process / Approach / Methods, Cyanobacteria < Taxonomic Group / Assemblage, Herbivory / grazing < Process / Approach / Methods, Other species interactions < Process / Approach / Methods, Crustacea < Taxonomic Group / Assemblage, Fresh waters < Habitat, Experimental ecology < Process / Approach / Methods

Context dependency of infectious disease: the cyanobacterium *Microcystis aeruginosa* decreases White Bacterial Disease in *Daphnia magna.*

MARLIES COOPMAN, KOENRAAD MUYLAERT, BENJAMIN LANGE, LIEN REYSERHOVE AND ELLEN DECAESTECKER

Aquatic Biology, KULeuven-Kulak, Kortrijk, Belgium

Correspondence: Marlies Coopman, Aquatic Biology, KULeuven-Kulak, 53 Etienne Sabbelaan, 8500 Kortrijk, Belgium. E-mail: marlies.coopman@kuleuven-kulak.be

Running headline: *Microcystis* decreases parasitism in *Daphnia*

Keywords: cyanobacteria, *Daphnia*, parasitism, antibacterial effect, toxic metabolites

Summary

1. We investigate whether an increase in the occurrence of cyanobacterial blooms affects zooplankton-parasite interactions. Cyanobacteria are expected to be of poor food quality for zooplankton hosts and are therefore expected to increase parasitism. Nevertheless, simultaneous exposure to both stressors may lead to different results, given the antibacterial secondary metabolites of cyanobacteria.
2. We exposed the zooplankter *Daphnia magna* to the cyanobacterial species *Microcystis aeruginosa* and the parasite that causes White Bacterial Disease in *D. magna*. Increased *M. aeruginosa* concentrations reduced the percentage of infected individuals and as such protected *D. magna* against parasitism. Interactions between *M. aeruginosa* and the parasite were antagonistic in terms of percentage of surviving *Daphnia*, total offspring per female and clutch size. Additional plating experiments showed a direct negative effect of *Microcystis* on bacterial growth.
3. The results suggest that changes in phytoplankton affect host-parasite interactions in zooplankton. Contrary to the prevailing paradigm that multiple stressors often induce additive or synergistic effects, we report an antagonistic effect of the presence of cyanobacterial stress on parasites in *Daphnia*. Thus, assessment of the outcome of host-parasite interactions needs to incorporate the environmental context.

Introduction

The intensification of human activities has profoundly affected biotic interactions in natural ecosystems. Often overlooked, host-parasite interactions are important in this context. In the long term, these interactions fuel biodiversity (Mouritsen & Poulin, 2005). and we need to understand how environmental changes affect host-parasite dynamics (Johnson & Carpenter; 2008, Lennon & Martiny, 2008; Duffy *et al.*, 2012). With respect to ecological effects on immunity, enhanced stressful conditions have often been associated with immunosuppression (Acevedo-Whitehouse & Duffus, 2009; Schmid-Hempel, 2011; Adamo, 2012). On the other hand, environmental changes that do not involve immunological responses may counteract the need for immune-based host responses (Parker *et al.*, 2011). There is thus a need to address the context dependency of infectious diseases (Ibelings *et al.*, 2011; Rohr *et al.*, 2011; Overholt *et al.*, 2012).

In this paper, we aim to unravel the effect of the cyanobacterium *Microcystis aeruginosa* on *Daphnia magna* parasitism. Cyanobacterial blooms have become a worldwide problem in many freshwater ecosystems (Codd, Morrison & Metcalf, 2005; Paerl & Huisman, 2008; Davis *et al.*, 2009; Kosten *et al.*, 2012). The negative effect of cyanobacteria on zooplankton and especially on *Daphnia* species is well documented (Ferrao, Azevedo & DeMott, 2000; Asselman *et al.,* 2012; Lemaire *et al.*, 2012). Many previous studies have focused on detrimental, toxic effects of microcystins (Demott, Zhang & Carmichael, 1991; Reinikainen, Ketola & Walls, 1994; Rohrlack *et al.*, 1999). However, not all negative effects are attributed to the presence of toxins (Berry *et al.*, 2008); recent studies also attribute it to the effects of low food quality (Martin-Creuzburg, von Elert & Hoffmann, 2008; von Elert, Zitt & Schwarzenberger, 2012). Cyanobacteria are low quality food for zooplankton in terms of biochemical composition because of the absence of long chain poly-unsaturated fatty acids

(PUFAs) and sterols (Muller-Navarra *et al.*, 2000; von Elert, Martin-Creuzburg & Le Coz, 2003; Martin-Creuzburg & von Elert, 2009) and the presence of protease inhibitors (Namikoshi & Rinehart, 1996; Rohrlack *et al.*, 2004; Schwarzenberger *et al.*, 2010).

Our focus here is the effect of food quality on zooplankton-parasite interactions and we therefore use a non-microcystin producing strain of *M. aeruginosa*. As well as their low food quality, cyanobacteria also harbour diverse secondary metabolites, including lipopeptides, amino acids, fatty acids, macrolides, amides that exhibit a wide range of bioactivities (e.g. antibacterial, antifungal, antialgal, antiprotozoan, antiviral) (Abed, Dobretsov & Sudesh, 2009; Singh *et al.*, 2011). Such secondary metabolites with biological activity have been found in *M. aeruginosa* (Ishida *et al.*, 1997; Abed *et al.*, 2009; Pradhan *et al.*, 2011). Nevertheless, *in vivo* experiments studying the effects on parasitism within hosts are extremely rare.

Our study animal is *Daphnia,* a keystone player in lake ecosystems because of its grazing effects on phytoplankton; *Daphnia* is also a laboratory model system (Miner *et al.*, 2012). Specifically, our study involves *Daphnia magna* because of its strong responses to changes in nutrient enrichment (Verreydt *et al.*, 2012) and associated *Microcystis* blooms. The responses of *Daphnia magna* to *Microcystis* exposure are well described (Nizan, Dimentman & Shilo, 1986; Schwarzenberger *et al.*, 2010; Lemaire *et al.*, 2012). *Daphnia magna* is also a model species for host-parasite interactions (Ebert, 2005; Ebert, 2011). The parasite we used causes White Bacterial Disease (WBD) and was selected for its strong virulence effects and its common occurrence in *Daphnia* populations in Western and Northern Europe (Decaestecker *et al.*, 2005; Ebert, 2005). Relatively few studies have investigated the role of food quality on *Daphnia* parasitism, but increasing evidence shows that changes in essential nutrients in food affect *Daphnia*-parasite interactions (Frost, Ebert

& Smith, 2008; Hall *et al.*, 2009; Frost *et al.*, 2010; Stjernman & Little, 2011; Aalto & Pulkkinen, 2013). *Daphnia* that are limited by food availability are considered to be more susceptible to parasites (Frost *et al.*, 2008; Schoebel, Wolinska & Spaak, 2010; Vale *et al.*, 2011). Given that cyanobacteria are considered to be of poor food quality (Muller-Navarra *et al.*, 2000; von Elert *et al.*, 2003; Martin-Creuzburg & von Elert, 2009), susceptibility of *Daphnia* to its parasite upon cyanobacterial exposure is expected to increase. The prevailing paradigm in multiple-stressor ecology predicts an increase in disease infectivity or virulence via weakened, immunosuppressed hosts (as detected in Coors *et al.*, 2008; Jansen *et al.*, 2011). Nevertheless, given the antibacterial effects of secondary metabolites detected *in vitro* in cyanobacteria, a decrease in bacterial parasitism would not be an unlikely outcome.

Methods

Isolation of *D. magna*, parasites and *M. aeruginosa*

We investigated the impact of a non-microcystin producing *M. aeruginosa* strain on *D. magna* upon exposure to the parasite that causes WBD. Due to increasing interest in non-microcystin related characteristics of cyanobacteria, such as food quality and the presence of bioactive secondary metabolites, a single celled, non-microcystin producing cyanobacterial strain of *M. aeruginosa* was used to unravel the cyanobacterial effect in the absence of toxic microcystins (*M. aeruginosa* CCAP1450/1, provided by the Culture Collection of Algae and Protozoa, UK, and isolated in 1948 from the highly productive Lake Mendota, Wisconsin, USA, where *M. aeruginosa* regularly dominates the lake) (Soranno, 1997). The strain was confirmed not to contain any microcystins. We performed polymerase chain reaction (PCR) amplification of three crucial genes of the microcystin synthetase cluster (mcyA, mcyB and mcyE: Neilan *et al.*, 1999; Hisbergues *et al.*, 2003; Vaitomaa *et al.*, 2003), but no amplified products were detected. The microcystin-producing *Microcystis aeruginosa* PCC7806 was used as a positive control. This strain is also available in other culture collections under different names (*M. aeruginosa* 1036, 1036AX, NIVA-CYA43, SAG 1450-1, UTCC 73, UTEX LB2061, UTEX 2061 *Microcystis* sp. PCC 7005 and *Synechocystis* sp. PCC 7005 and ATCC 27153) and different research groups have demonstrated its inability to produce microcystins (reviewed by Lyra *et al.*, 2001). *M. aeruginosa* strains were cultured in modified WC medium in 1 L bottles that were aerated and stirred (Guillard & Lorenzen, 1972, the medium did not contain Tris). Stocks of *D. magna*, *M. aeruginosa* and *Scenedesmus obliquus* were cultured in a climate chamber at 20±2°C with a light:dark cycle of 16:8 h. Experiments were performed under the same conditions.

The *D. magna* clone, originally obtained by hatching dormant eggs, was isolated from the sediment of a pond in Heverlee, Belgium (OM2, "Abdij van 't Park", 50°51'47.67"N; 4°43'16.36"E). This *D. magna* population has been well described with respect to its parasitic interactions (Decaestecker *et al.*, 2005; Decaestecker *et al.*, 2007; Decaestecker *et al.*, 2013). *Daphnia* clone 16.1 was chosen because of its good performance in laboratory conditions and its sensitivity to the parasite. Before running the experiment, maternal effects were controlled by culturing fifteen iso-female lines separately under standardized conditions (20±2°C with a light:dark cycle of 16:8h) for three generations. Lineages were fed daily with 1.5 mg C L^{-1} *Scenedesmus obliquus* and medium was refreshed three times a week. *Daphnia* culturing and experiments were performed in ADaM medium (Kluttgen *et al.*, 1994).

We used the parasite that causes White Bacterial Disease (WBD) in *D. magna*. WBD, also known as White Fat Cell Disease (WFD), is caused by a small coccoid parasite, most likely a bacterium that infects the adipose tissue, resulting in a bright white reflection with a greenish shine from infected tissue that is only visible in reflected light (Ebert, 2005). The infection is virulent and is transmitted horizontally (Decaestecker *et al.*, 2005). Infected females were collected from another *D. magna* population than the clone that was used in the final experiment, to exclude pre-adaptation. The parasite was isolated from a pond in Zonhoven, Belgium (lake 21, 50° 59' 12.72" N en 5° 20' 35.56" E) and kept in a 400 L container with non-infected *D. magna* individuals from the same pond. Upon establishment of infections, infected *Daphnia* individuals were picked from the 400 L container and kept at 4°C until the experiment was performed. In the parasite treatment, *Daphnia* were exposed to a solution of homogenized infected *Daphnia* (3x25 cadavers in 3x5 mL). Parasite exposure consisted of one infected individual for three females to be infected. An equal number of healthy individuals was homogenized for use in the control treatment.

***In vivo Daphnia* exposure to *Microcystis* and WBD**

Neonates (<24 h old) from the second brood of the third generation of the maternal lines were isolated and five individuals were pooled and placed in a 200 mL experimental jar (design according to Lemaire *et al.*, 2012). Five replicates were performed per treatment combination, each from a different mother line to control for maternal effects and interdependency of the different replicates. Neonates were fed daily 5 mg C L^{-1} of a mixture of *M. aeruginosa* and *S. obliquus* in concentrations of 0%, 10%, 20%, 50%, 70% and 100% *M. aeruginosa*. This scale was chosen based on results of earlier pilot experiments. In total, 60 experimental jars were involved (6 concentrations x 2 parasite treatments (= with and without parasite) x 5 replicates). The medium was refreshed daily until day 4. From day five, female *D. magna* juveniles were exposed for five days to a mixture of homogenized WBD-infected *Daphnia* tissue. On day five, the *Daphnia* were exposed to the parasite or placebo solution in 50 mL, at day six and seven in 100 mL and at day eight and nine in 200 mL. Addition of the parasite or placebo solution was spread over three consecutive days and the added volume was recalculated every day to make sure that each living *Daphnia* was exposed to an equal number of parasites. From day ten until the end of the experiment, the medium was refreshed daily. Survival was measured daily as the number of surviving individuals out of five *Daphnia* in each jar. The number of offspring produced and the number of WBD-infected individuals were recorded daily. On day 21, two females from each microcosm were randomly chosen, measured (as in Coors & De Meester, 2008) and their clutch size was recorded.

In vitro test of the antibacterial effect of _Microcystis_

To confirm a potential direct negative effect of the cyanobacterial food on the *D. magna* parasite, we tested antimicrobial activity of the extracts of *M. aeruginosa* and *S. obliquus* using a paper disc diffusion assay. As no standard cultures and plating techniques are yet available for WBD, plating experiments were carried out using *Escherichia coli* (NovaBlue Singles™ Competent Cells, Novagen, EMD Biosciences, Inc, Darmstadt, Germany). 10 mg C of the *M. aeruginosa* or *S. obliquus* strain was concentrated by centrifugation and lysed using five freeze-thaw cycles. Sterilized filter paper discs (6 mm) were saturated with the filtered extract (0.22 µm) and placed on a monolayer of *E. coli* on agar. Paper discs saturated with a 3 mg mL^{-1} tetracycline solution were used as positive controls. Cultures were incubated for 20 hours at 37°C before diameters of the inhibition zones surrounding the disc were measured (Andrews, 2001).

Statistical analysis

Generalized Linear Models (GLMs) were used to investigate the effect of the parasite exposure and the effect of increasing *M. aeruginosa* concentration (0%, 10%, 20%, 50%, 70%, 100%, categorical data). Percentage surviving *Daphnia* was analyzed as binomially-distributed data, as the ratio of surviving *Daphnia* to the number of dead *Daphnia*. Body size data (mean value for each experimental jar) were analyzed as normally-distributed data. Total offspring per female was calculated as the sum of daily offspring per jar divided by total surviving *Daphnia*, since all *Daphnia* in this experiment were female. Total offspring per female was also analyzed as normally-distributed data. Clutch size (eggs per female at the end of the experiment) was treated as count data and analyzed by a Poisson distribution (mean value for each experimental jar). Time to first clutch (i.e. the time when the first

juveniles appeared in the jar) was analyzed by survival analysis, specifically the Cox proportional hazards model (Efron method). In this analysis, surviving females that had not reproduced by the end of the experiment were taken into account as right-sensored data. Percentage infected *Daphnia* per jar was analyzed as binomially-distributed data. In all analyses the ratio of the residual deviance to the degrees of freedom was verified to be one. If not, data were corrected for over- or underdispersion. Then the dispersion parameter was not fixed at one, but estimated by moments. To compare the influence of the parasite (control versus exposed) at different *S. obliquus*/*M. aeruginosa* mixtures, a Tukey post hoc analysis was performed for the response variables percentage surviving *Daphnia*, body size, clutch size, time to first clutch and total offspring per female.

No statistical analyses were performed on the antibacterial activity of tetracycline and the lysates of *Microcystis* and *Scenedesmus* since only the appearance of a halo, showing antibacterial effects of the solution on *E. coli*, was important for this experiment. All tests were performed using the free statistical tool pack R version 2.10. (http://www.r-project.org/).

Results

The results of our experiment almost always showed a significant negative effect when the two stressors were applied separately. *M. aeruginosa* negatively affected the *D. magna* clone: the percentage of surviving *Daphnia*, body size and clutch size decreased (Fig. 1a,b,c, Table 1) while time to first clutch increased with an increasing *M. aeruginosa* concentration (Fig. 1d, Table 1), resulting in a significant decrease in total offspring per female with increasing *M. aeruginosa* concentration (Fig 1e, Table 1). Exposure to WBD had a negative effect on *Daphnia* by reducing the percentage of surviving *Daphnia* (marginally significant) and clutch size (Fig. 1a,c, Table 1) but had a positive effect on *Daphnia* body size and total offspring per female (Fig. 1b,e Tables 1). No parasite effect was observed for time to first clutch (Fig. 1d, Table 1).

When *D. magna* was simultaneously exposed to both stressors, interaction effects (i.e. antagonistic effects between the two stressors) could be detected, especially between 0% and 20% *M. aeruginosa*. There were significant interaction effects between the *M. aeruginosa* and WBD treatment on *Daphnia* survival (percentage surviving *Daphnia*), total offspring per female and clutch size (Table 1). There was no interaction effect for body size or time to first clutch (Tables 1). The negative effect of WBD on percentage *Daphnia* survival and clutch size was present when no *M. aeruginosa* were present in the food treatment, but disappeared at 20% *M. aeruginosa* (Fig. 1a,c, Table 2).

No interaction was found for time to first clutch over the range of 0 to 100% *M. aeruginosa* but a reduction of the negative *Microcystis* effect was observed upon simultaneous WBD exposure and 20% *M. aeruginosa* in the food treatment (Fig. 1d, Table 2). Non-WBD exposed *Daphnia* produced their first brood later when fed with 20% *M. aeruginosa* in comparison with the 0% *M. aeruginosa* food treatment (Tukey post hoc

analysis of non-WBD exposed *Daphnia* between 0% and 20% *Microcystis*: $p = 0.025$). Upon exposure to WBD, there was no difference in time to first brood between the 0% and the 20% *M. aeruginosa* food treatments (Tukey post hoc analysis of WBD exposed *Daphnia* between 0% and 20% *Microcystis*: $p = 0.984$). Further, total offspring per female was higher in the WBD exposed assay compared to the placebo treatment at 20% *M. aeruginosa* in the food treatment (Fig. 1e, Table 2). This is probably due to the earlier first brood at 20% *M. aeruginosa* (Fig. 1d, Table 2).

There was a reduction in the percentage infected *Daphnia* upon increasing *M. aeruginosa* concentration in the food (Fig. 1f, GLM for binomially-distributed data: likelihood ratio 24.464, degrees of freedom 5, $p < 0.001$). Additionally, a paper disc diffusion bioassay demonstrated direct antibacterial activity of the *M. aeruginosa* lysate on *E. coli* (Table 3). The lysate of *S. obliquus* did not show this activity.

Discussion

Both the cyanobacterial strain *M. aeruginosa* and the parasite WBD individually induced negative fitness effects in *D. magna* and can thus be considered as biotic stressors. In view of its negative effects on life history traits, *M. aeruginosa* has generally been considered to be a biotic stressor for *D. magna* (Ferrao *et al.*, 2000; Asselman *et al.*, 2012; Lemaire *et al.*, 2012). The present study confirms that these negative effects are indeed present upon individual exposure of *Daphnia* to *M. aeruginosa*. The negative effects associated with *M. aeruginosa* can be assigned to toxic substances such as microcystins (Demott *et al.*, 1991) or gamma-linolenic acid (Reinikainen *et al.*, 2001) or poor food quality such as the absence of sterols and certain essential PUFAs (Martin-Creuzburg *et al.*, 2008) or the presence of protease inhibitors (von Elert *et al.*, 2012). The frequently observed mechanical obstruction of *Daphnia's* filtering apparatus (Lampert, 1987; De Bernardi & Giussani, 1990; DeMott, Gulati & Van Donk, 2001) does not apply here because the *Microcystis* strain that we used consisted only of single cells.

We detected an antagonistic combined effect of the stress of *M. aeruginosa* and WBD in this particular association of host clone and parasite strain. This is contrary to our original expectation based on the prevailing paradigm that multiple stressors (especially when toxicants are involved) are assumed to induce mostly additive or synergistic effects (Coors *et al.*, 2008; Sures, 2008; Holmstrup *et al.*, 2010). *Microcystis* and cyanobacterial stress in general can be considered as an exception, given the antibacterial properties of secondary metabolites, which have so far mainly been investigated *in vitro* (Ishida *et al.*, 1997; Abed *et al.*, 2009; Pradhan *et al.*, 2011). One research group has detected negative effects of cyanobacteria on parasitism in tomato plants *in vivo* (Khan *et al.*, 2005; Khan *et al.*, 2007). Antibacterial effects may make cyanobacteria an attractive source for

pharmacologically active compounds (reviewed by Abed *et al.*, 2009; Rastogi & Sinha, 2009). The production of such substances may confer a survival advantage by aiding the cyanobacterium in competition with other bacteria or algae (Ishida *et al.*, 1997; Singh *et al.*, 2001; Leflaive & Ten-Hage, 2007). Note that our *M. aeruginosa* strain has been cultured for almost seventy years in a laboratory. In this strain, competition with other algae or cyanobacteria is absent, but other bacteria may be present (non-axenic culture, see Culture Collection of Algae and Protozoa, UK). However, as only the lysate was tested *in vitro* against *E. coli*, the specific substances that are responsible for the antibacterial effect are not known.

Our study also revealed a negative effect of *Microcystis* on the parasite. This antibacterial result cannot, however, be generalized because our *in vivo* study is based on a single parasite species-*Microcystis* strain exposed to a single *D. magna* clone. The *in vitro* response did, however, also show an effect of the *M. aeruginosa* strain on *E. coli*. We therefore hypothesize that feeding on antibacterial cyanobacteria may help reduce infections in *Daphnia* in the field. The parasite that causes White Bacterial Disease has been found in ponds where *M. aeruginosa* blooms occur (OM2, Heverlee, Belgium), but to our knowledge co-occurrence of *M. aeruginosa* and WBD has never been reported.

Our experiment has also demonstrated that, upon exposure to both stressors, *Daphnia* begins reproducing earlier. While fecundity compensation through a reproduction shift upon infection had been detected in *Daphnia* before (Ebert *et al.*, 2004; Chadwick & Little, 2005), the fecundity compensation reported here only occurred when both stressors were present. The time to start reproducing was not delayed in the presence of both stressors as was the case upon *M. aeruginosa* exposure only. Together with an increase in clutch size, which was probably due to a decreasing parasite effect, this resulted in an

increase in total offspring per surviving female.

Rather than being an enhanced immune response elicited in *Daphnia* by the presence of cyanobacteria, the detected antibacterial activity is more likely a direct effect of the cyanobacteria on the WBD parasite. Nevertheless, we cannot exclude a role for enhanced immuno-competence in *Daphnia* (immuno-enhancement has been reported upon short stress exposure; Demas, Adamo & French, 2011). Much attention has been devoted in the past decade to the effects of environmental constraints on the role immune systems play in determining host fitness in the wild (Lochmiller & Deerenberg, 2000; Woodhams *et al.*, 2008; Sadd & Schmid-Hempel, 2009). In general, and with respect to invertebrates, immuno-suppression is expected in stressful conditions because of reduced investment in mounting an immune response (Schmid-Hempel, 2011; Adamo, 2012). In this context it is interesting to speculate whether the gut microbiota of *Daphnia* may also have mediated the antibacterial response (Rengpipat *et al.*, 2000; Dillon *et al.*, 2005) or have stimulated mortality in infected *Daphnia*.

It is known that *Daphnia* can avoid *Microcystis*, probably based on taste (Rohrlack *et al.*, 2001; Tillmanns, Burton & Pick, 2011). In the presence of single and non-microcystin producing *Microcystis* cells, *Daphnia* reduces its ingestion rate (Tillmanns *et al.*, 2011). In addition to low food quality of *Microcystis*, this may help explain reduced survival in the absence of the parasites. Consequently, as spores of WBD are most likely taken up by the filter apparatus of *Daphnia*, the ingestion of WBD spores will also be reduced in the presence of *Microcystis*. This may explain the decline in the percentage infected *Daphnia* upon increasing *M. aeruginosa* concentration in the food. However, other factors may also come into play, as the reduced ingestion rate may not completely explain *Daphnia* survival when exposed to WBD. At 20% *Microcystis*, food and spores can still be filtered and *Daphnia* still

become infected, but the consequent death penalty disappears from this concentration onwards. It seems that infection is kept under control from 20% *Microcystis* onwards. *Daphnia* still get infected, but survive the infection. It is possible that *Daphnia* develops resistance against *Microcystis* and is therefore also more defended against parasite spores (cross-reactivity, Schmid-Hempel, 2011). Another explanation could be the suppression of disease by the antibacterial metabolites produced by *Microcystis* (and an associated reduction in virulence through a reduction in bacterial growth). However, as decreased infection rates were also found with phosphorus-limited food (Frost *et al.*, 2008) follow-up studies are needed to disentangle the effects of low food quality, immune host responses and the direct effect of antibacterial substances.

Acknowledgments

We are grateful to collaborating research groups (Hans Deckmyn, Zeger Debyser, Melanie Gerard) at the IRF Life Sciences Kulak for practical microbial assistance in the laboratory. We thank Hans Pottel for his statistical advice and Dominik Martin-Creuzburg, Luc De Meester and four anonymous referees for comments on earlier versions of the manuscript. We thank Liesbet Heyvaert for linguistic corrections. Funding was provided by the research projects START1/08/019, Belspo IAP project P7/4 and the Centre of Excellence PF/2010/007 of the KULeuven Research Fund.

References

Aalto, S.L. & Pulkkinen, K. (2013) Food stoichiometry affects the outcome of *Daphnia*-parasite interaction. *Ecology and Evolution,* **3,** 1266-1275.

Abed, R.M.M., Dobretsov, S. & Sudesh, K. (2009) Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology,* **106,** 1-12.

Acevedo-Whitehouse, K. & Duffus, A.L.J. (2009) Effects of environmental change on wildlife health. *Philosophical Transactions of the Royal Society B-Biological Sciences,* **364,** 3429-3438.

Adamo, S.A. (2012) The effects of the stress response on immune function in invertebrates: An evolutionary perspective on an ancient connection. *Hormones and Behavior,* **62,** 324-330.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* **48,** 5-16.

Asselman, J., Glaholt, S.P., Smith, Z., Smagghe, G., Janssen, C.R., Colbourne, J.K. *et al.* (2012) Functional characterization of four metallothionein genes in *Daphnia pulex* exposed to environmental stressors. *Aquatic Toxicology,* **110,** 54-65.

Berry, J.P., Gantar, M., Perez, M.H., Berry, G. & Noriega, F.G. (2008) Cyanobacterial toxins as allelochemicals with potential applications as algaecides, herbicides and insecticides. *Marine Drugs,* **6,** 117-146.

Chadwick, W. & Little, T.J. (2005) A parasite-mediated life-history shift in *Daphnia magna*. *Proceedings of the Royal Society B-Biological Sciences,* **272,** 505-509.

Codd, G.A., Morrison, L.F. & Metcalf, J.S. (2005) Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology,* **203,** 264-272.

Coors, A. & De Meester, L. (2008) Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. *Journal of Applied Ecology,* **45,** 1820-1828.

Coors, A., Decaestecker, E., Jansen, M. & De Meester, L. (2008) Pesticide exposure strongly enhances parasite virulence in an invertebrate host model. *Oikos,* **117,** 1840-1846.

Davis, T.W., Berry, D.L., Boyer, G.L. & Gobler, C.J. (2009) The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae,* **8,** 715-725.

De Bernardi, R. & Giussani, G. (1990) Are blue-green algae a suitable food for zooplankton – an overview. *Hydrobiologia,* **200,** 29-41.

Decaestecker, E., De Gersem, H., Michalakis, Y. & Raeymaekers, J.A.M. (2013) Damped long-term host-parasite Red Queen coevolutionary dynamics: a reflection of dilution effects? *Ecology Letters*, **16**, 1455-1462.

Decaestecker, E., Declerck, S., De Meester, L. & Ebert, D. (2005) Ecological implications of parasites in natural *Daphnia* populations. *Oecologia,* **144,** 382-390.

Decaestecker, E., Gaba, S., Raeymaekers, J.A.M., Stoks, R., Van Kerckhoven, L., Ebert, D. *et al.* (2007) Host-parasite 'Red Queen' dynamics archived in pond sediment. *Nature,* **450,** 870-U816.

Demas, G.E., Adamo, S.A. & French, S.S. (2011) Neuroendocrine-immune crosstalk in vertebrates and invertebrates: implications for host defence. *Functional Ecology,* **25,** 29-39.

Demott, W.R., Gulati, R.D. & Van Donk, E. (2001) *Daphnia* food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. *Limnology and Oceanography,* **46,** 2054-2060.

Demott, W.R., Zhang, Q.X. & Carmichael, W.W. (1991) Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnology and Oceanography,* **36,** 1346-1357.

Dillon, R.J., Vennard, C.T., Buckling, A. & Charnley, A.K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters,* **8,** 1291-1298.

Duffy, M.A., Ochs, J.H., Penczykowski, R.M., Civitello, D.J., Klausmeier, C.A. & Hall, S.R. (2012) Ecological Context Influences Epidemic Size and Parasite-Driven Evolution. *Science,* **335,** 1636-1638.

Ebert, D. (2005) *Ecology, Epidemiology, and Evolution of Parasitism in* Daphnia. National Center for Biotechnology Information (US), Bethesda (MD).

Ebert, D. (2011) A Genome for the Environment. *Science,* **331,** 539-540.

Ebert, D., Carius, H.J., Little, T. & Decaestecker, E. (2004) The evolution of virulence when parasites cause host castration and gigantism. *American Naturalist,* **164,** S19-S32.

Ferrao, A.S., Azevedo, S. & Demott, W.R. (2000) Effects of toxic and non-toxic cyanobacteria on the life history of tropical and temperate cladocerans. *Freshwater Biology,* **45,** 1-19.

Frost, P.C., Ebert, D., Larson, J.H., Marcus, M.A., Wagner, N.D. & Zalewski, A. (2010) Transgenerational effects of poor elemental food quality on *Daphnia magna*. *Oecologia,* **162,** 865-872.

Frost, P.C., Ebert, D. & Smith, V.H. (2008) Responses of a bacterial pathogen to phosphorus limitation of its aquatic invertebrate host. *Ecology,* **89,** 313-318.

Guillard, R.R. & Lorenzen, C.J. (1972) Yellow-green algae with chlorophyllide C. *Journal of Phycology,* **8,** 10-14.

Hall, S.R., Knight, C.J., Becker, C.R., Duffy, M.A., Tessier, A.J. & Caceres, C.E. (2009) Quality matters: resource quality for hosts and the timing of epidemics. *Ecology Letters,* **12,** 118-128.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. & Borner, T. (2003) PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. *Archives of Microbiology,* **180,** 402-410.

Holmstrup, M., Bindesbol, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Kohler, H.R. *et al.* (2010) Interactions between effects of environmental chemicals and natural stressors: A review. *Science of the Total Environment,* **408,** 3746-3762.

Ibelings, B.W., Gsell, A.S., Mooij, W.M., Van Donk, E., Van Den Wyngaert, S. & Domis, L.N.D. (2011) Chytrid infections and diatom spring blooms: paradoxical effects of climate warming on fungal epidemics in lakes. *Freshwater Biology,* **56,** 754-766.

Ishida, K., Matsuda, H., Murakami, M. & Yamaguchi, K. (1997) Kawaguchipeptin B, an antibacterial cyclic undecapeptide from the cyanobacterium *Microcystis aeruginosa*. *Journal of Natural Products,* **60,** 724-726.

Jansen, M., Stoks, R., Coors, A., Van Doorslaer, W. & De Meester, L. (2011) Collateral damage: rapid exposure-induced evolution of pesticide resistance leads to increased susceptibility to parasites. *Evolution,* **65,** 2681-2691.

Johnson, P.T.J. & Carpenter, S.R. (2008) Influence of Eutrophication on Disease in Aquatic Ecosystems: Patterns, Processes, and Predictions. In: *Infectious Disease Ecology: The effects of Ecosystems on Disease and of Disease on Ecosystems.* (Eds R.S. Ostfeld, F. Keesing & V.T. Eviner), pp. 71-99. Princeton University Press, Princeton.

Khan, Z., Kim, Y.H., Kim, S.G. & Kim, H.W. (2007) Observations on the suppression of root-knot nematode (*Meloidogyne arenaria*) on tomato by incorporation of cyanobacterial

powder (*Oscillatoria chlorina*) into potting field soil. *Bioresource Technology,* **98,** 69-73.

Khan, Z., Park, S.D., Shin, S.Y., Bae, S.G., Yeon, I.K. & Seo, Y.J. (2005) Management of *Meloidogyne incognita* on tomato by root-dip treatment in culture filtrate of the blue-green alga, *Microcoleus vaginatus*. *Bioresource Technology,* **96,** 1338-1341.

Kluttgen, B., Dulmer, U., Engels, M. & Ratte, H.T. (1994) ADaM, an artificial freshwater for the culture of zooplankton. *Water Research,* **28,** 743-746.

Kosten, S., Huszar, V.L.M., Bécares, E., Costa, L.S., Van Donk, E., Hansson, L.-A. *et al.* (2012) Warmer climates boost cyanobacterial dominance in shallow lakes. *Global Change Biology,* **18,** 118-126.

Lampert, W. (1987) Laboratory studies on zooplankton-cyanobacteria interactions. *New Zealand Journal of Marine and Freshwater Research,* **21,** 483-490.

Leflaive, J. & Ten-Hage, L. (2007) Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. *Freshwater Biology,* **52,** 199-214.

Lemaire, V., Brusciotti, S., Van Gremberghe, I., Vyverman, W., Vanoverbeke, J. & De Meester, L. (2012) Genotype genotype interactions between the toxic cyanobacterium *Microcystis* and its grazer, the waterflea *Daphnia*. *Evolutionary Applications,* **5,** 168-182.

Lennon, J.T. & Martiny, J.B.H. (2008) Rapid evolution buffers ecosystem impacts of viruses in a microbial food web. *Ecology Letters,* **11,** 1178-1188.

Lochmiller, R.L. & Deerenberg, C. (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos,* **88,** 87-98.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L. *et al.* (2001) Molecular characterization of planktic cyanobacteria of *Anabaena, Aphanizomenon, Microcystis* and *Planktothrix* genera. *International Journal of Systematic and Evolutionary Microbiology,* **51,** 513-526.

Martin-Creuzburg, D. & Von Elert, E. (2009) Good food versus bad food: the role of sterols and polyunsaturated fatty acids in determining growth and reproduction of *Daphnia magna*. *Aquatic Ecology,* **43,** 943-950.

Martin-Creuzburg, D., Von Elert, E. & Hoffmann, K.H. (2008) Nutritional constraints at the cyanobacteria-*Daphnia magna* interface: The role of sterols. *Limnology and Oceanography,* **53,** 456-468.

Miner, B.E., De Meester, L., Pfrender, M.E., Lampert, W. & Hairston, N.G. (2012) Linking genes to communities and ecosystems: *Daphnia* as an ecogenomic model. *Proceedings of the Royal Society B-Biological Sciences,* **279,** 1873-1882.

Mouritsen, K.N. & Poulin, R. (2005) Parasites boosts biodiversity and changes animal community structure by trait-mediated indirect effects. *Oikos,* **108,** 344-350.

Muller-Navarra, D.C., Brett, M.T., Liston, A.M. & Goldman, C.R. (2000) A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature,* **403,** 74-77.

Namikoshi, M. & Rinehart, K.L. (1996) Bioactive compounds produced by cyanobacteria. *Journal of Industrial Microbiology & Biotechnology,* **17,** 373-384.

Neilan, B.A., Dittmann, E., Rouhiainen, L., Bass, R.A., Schaub, V., Sivonen, K. *et al.* (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. *Journal of Bacteriology,* **181,** 4089-4097.

Nizan, S., Dimentman, C. & Shilo, M. (1986) Acute toxic effects of the cyanobacterium *Microcystis aeruginosa* on *Daphnia magna*. *Limnology and Oceanography,* **31,** 497-502.

Overholt, E.P., Hall, S.R., Williamson, C.E., Meikle, C.K., Duffy, M.A. & Caceres, C.E. (2012) Solar radiation decreases parasitism in *Daphnia*. *Ecology Letters,* **15,** 47-54.

Paerl, H.W. & Huisman, J. (2008) Climate - Blooms like it hot. *Science,* **320,** 57-58.

Parker, B.J., Barribeau, S.M., Laughton, A.M., De Roode, J.C. & Gerardo, N.M. (2011) Non-immunological defense in an evolutionary framework. *Trends in Ecology & Evolution,* **26,** 242-248.

Pradhan, J., Sahu, S., Marhual, N.P., Mishra, B.K. & Das, B.K. (2011) Antibacterial properties of freshwater *Microcystis aeruginosa* (Kutz) to bacterial pathogen-a comparative study of bacterial bioassays. *Indian Journal of Animal Sciences,* **81,** 1266-1271.

Rastogi, R.P. & Sinha, R.P. (2009) Biotechnological and industrial significance of cyanobacterial secondary metabolites. *Biotechnology Advances,* **27,** 521-539.

Reinikainen, M., Ketola, M. & Walls, M. (1994) Effects of the concentrations of toxic *Microcystis aeruginosa* and an alternative food on the survival of *Daphnia pulex*. *Limnology and Oceanography,* **39,** 424-432.

Reinikainen, M., Meriluoto, J.A.O., Spoof, L. & Harada, K. (2001) The toxicities of a polyunsaturated fatty acid and a microcystin to *Daphnia magna*. *Environmental Toxicology,* **16,** 444-448.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S. & Menasaveta, P. (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (Bacillus S11). *Aquaculture,* **191,** 271-288.

Rohr, J.R., Dobson, A.P., Johnson, P.T.J., Kilpatrick, A.M., Paull, S.H., Raffel, T.R. *et al.* (2011) Frontiers in climate change-disease research. *Trends in Ecology & Evolution,* **26,** 270-277.

Rohrlack, T., Christoffersen, K., Kaebernick, M. & Neilan, B.A. (2004) Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. *Applied and Environmental Microbiology,* **70,** 5047-5050.

Rohrlack, T., Dittmann, E., Borner, T. & Christoffersen, K. (2001) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied and Environmental Microbiology,* **67,** 3523-3529.

Rohrlack, T., Dittmann, E., Henning, M., Borner, T. & Kohl, J.G. (1999) Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. *Applied and Environmental Microbiology,* **65,** 737-739.

Sadd, B.M. & Schmid-Hempel, P. (2009) Principles of ecological immunology. *Evolutionary Applications,* **2,** 113-121.

Schmid-Hempel (2011) *Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology and Genetics,* Oxford University Press Inc., New York.

Schoebel, C.N., Wolinska, J. & Spaak, P. (2010) Higher parasite resistance in *Daphnia* populations with recent epidemics. *Journal of Evolutionary Biology,* **23,** 2370-2376.

Schwarzenberger, A., Zitt, A., Kroth, P., Mueller, S. & Von Elert, E. (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC Physiology,* **10,** 6.

Singh, D.P., Tyagi, M.B., Kumar, A. & Thakur, J.K. (2001) Antialgal activity of a hepatotoxin-producing cyanobacterium, *Microcystis aeruginosa*. *World Journal of Microbiology & Biotechnology,* **17,** 15-22.

Singh, R.K., Tiwari, S.P., Rai, A.K. & Mohapatra, T.M. (2011) Cyanobacteria: an emerging source for drug discovery. *Journal of Antibiotics,* **64,** 401-412.

Soranno, P.A. (1997) Factors affecting the timing of surface scums and epilimnetic blooms of blue-green algae in a eutrophic lake. *Canadian Journal of Fisheries and Aquatic Sciences,* **54,** 1965-1975.

Stjernman, M. & Little, T.J. (2011) Genetic variation for maternal effects on parasite susceptibility. *Journal of Evolutionary Biology,* **24,** 2357-2363.

Sures, B. (2008) Environmental Parasitology. Interactions between parasites and pollutants in the aquatic environment. *Parasite-Journal De La Societe Francaise De Parasitologie,* **15,** 434-438.

Tillmanns, A.R., Burton, S.K. & Pick, F.R. (2011) *Daphnia* Pre-Exposed to Toxic *Microcystis* Exhibit Feeding Selectivity. *International Review of Hydrobiology,* **96,** 20-28.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. *et al.* (2003) Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. *Applied and Environmental Microbiology,* **69,** 7289-7297.

Vale, P.F., Wilson, A.J., Best, A., Boots, M. & Little, T.J. (2011) Epidemiological, Evolutionary, and Coevolutionary Implications of Context-Dependent Parasitism. *American Naturalist,* **177,** 510-521.

Verreydt, D., De Meester, L., Decaestecker, E., Villena, M.-J., Van Der Gucht, K., Vannormelingen, P. *et al.* (2012) Dispersal-mediated trophic interactions can

generate apparent patterns of dispersal limitation in aquatic metacommunities. *Ecology Letter*, **15**, 218-226.

Von Elert, E., Martin-Creuzburg, D. & Le Coz, J.R. (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London Series B-Biological Sciences,* **270,** 1209-1214.

Von Elert, E., Zitt, A. & Schwarzenberger, A. (2012) Inducible tolerance to dietary protease inhibitors in *Daphnia magna*. *Journal of Experimental Biology,* **215,** 2051-2059.

Woodhams, D.C., Alford, R.A., Briggs, C.J., Johnson, M. & Rollins-Smith, L.A. (2008) Life-history trade-offs influence disease in changing climates: Strategies of an amphibian pathogen. *Ecology,* **89,** 1627-1639.

Tables

Table 1 Results of Generalized Linear Models (GLM) testing for the effect of parasite exposure (P) and *M. aeruginosa* concentration (C) in the food on percentage surviving *Daphnia*, body size, clutch size and total offspring per female. Time to first clutch was analyzed by a Cox proportional hazards model.

Response variable	Effect	Likelihood ratio	Degrees of Freedom	p-value
Percentage surviving *Daphnia*	C	49.376	5	<0.001
	P	13.647	1	<0.001
	P x C	18.616	5	0.002
Body size	C	55.366	5	<0.001
	P	7.760	1	<0.001
	P x C	2.807	5	0.730
Clutch size	C	33.184	5	<0.001
	P	43.319	1	<0.001
	P x C	23.611	5	<0.001
Time to first clutch	C	33.065	5	<0.001
	P	1.135	1	0.287
	P x C	9.048	5	0.107
Total offspring per female	C	119.060	5	<0.001
	P	7.592	1	0.006
	P x C	24.297	5	<0.001

Table 2 Results of Tukey post hoc tests analyzing different response variables between WBD exposed and non-exposed *D. magna* females fed with mixtures of 0/100% and 20/80% *M. aeruginosa/S. obliquus.*

Response variable	Percentage *Microcystis*	Estimated value	Standard error	Z-value	p-value
Percentage surviving *Daphnia*	0%	3.932	1.516	2.593	0.056
	20%	0.399	0.870	0.459	0.998
Body size	0%	0.378	0.136	2.786	0.032
	20%	0.355	0.128	2.775	0.033
Clutch size	0%	-2.152	0.433	-4.973	<0.001
	20%	-0.331	0.215	-1.540	0.547
Time to first clutch	0%	-0.748	0.703	-1.064	0.867
	20%	2.452	0.756	3.243	0.007
Total offspring per female	0%	-8.900	3.230	-2.755	0.035
	20%	11.413	3.230	3.533	0.002

Table 3 Antibacterial activity of tetracycline (3 mg mL^{-1}) and the lysate of *Scenedesmus* and *Microcystis* against *E. coli* using the disc diffusion bioassay.

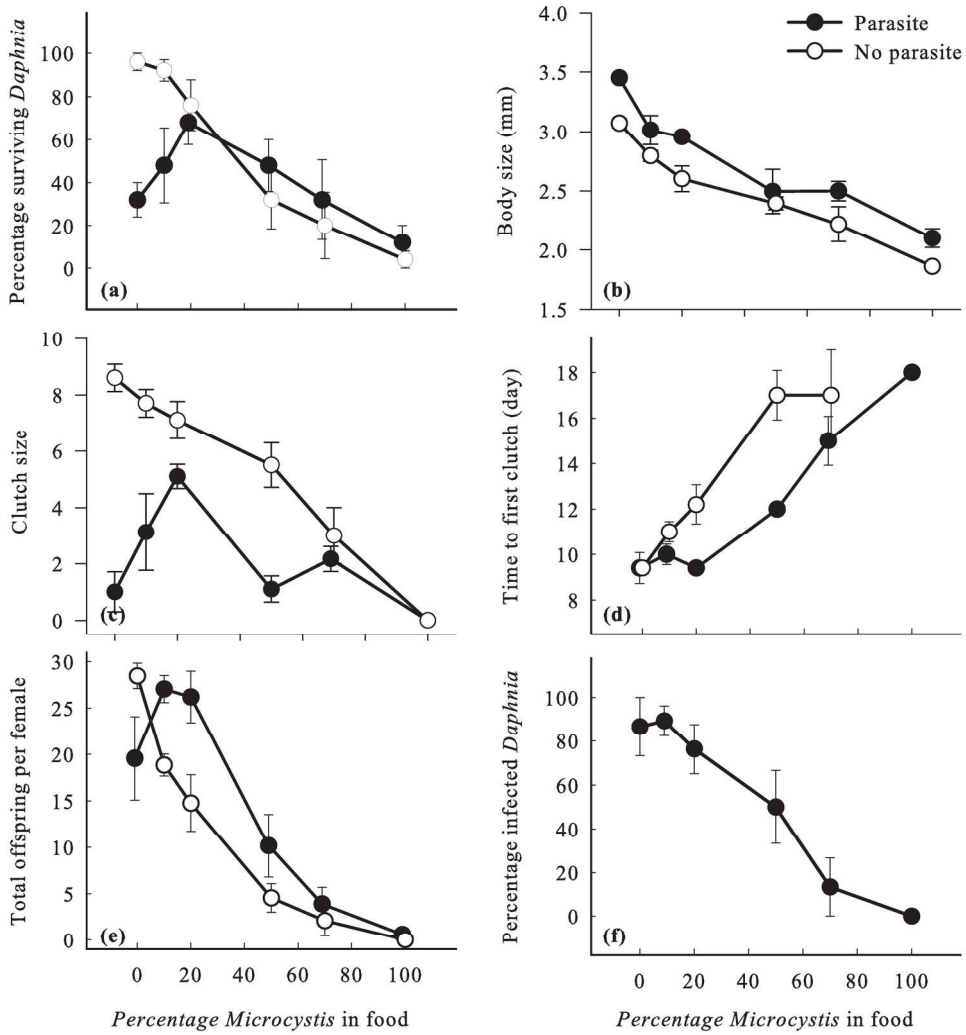
Substrate	Inhibition zone
Tetracycline	YES
Lysate of *S. obliquus*	NO
Lysate of *M. aeruginosa*	YES

Figure legends

Fig. 1 (a) Percentage surviving *Daphnia*, (b) body size and (c) clutch size of *Daphnia* surviving on day 21 of the experiment, (d) time to first clutch, (e) total offspring per female, and (f) percentage of *D. magna* females infected with White Bacterial Disease as a function of the *M. aeruginosa* - *S. obliquus* mixtures. Means are given and error bars indicate one standard error.

Illustrations

Fig. 1 See attachment



182x200mm (300 x 300 DPI)